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IDENTIFICATION OF A SEX PHEROMONE COMPONENT FOR THE BLUEBERRY LEAFMINER, *Caloptilia porphyretica*

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Abstract—Coupled gas chromatographic–electroantennographic detection (GC–EAD) of both gland extracts and effluvial collections from female blueberry leafminer, *Caloptilia porphyretica* Braun (Lepidoptera: Gracillariidae), showed that females produced a single EAD-active compound. The amount of the compound collected from virgin female *C. porphyretica* was below GC and mass spectrometry (MS) detection thresholds, even with highly concentrated gland extracts (~150 female equivalent). (*E*)-11-Hexadecenal (*E*11-16:Ald) was determined to be a sex pheromone component mainly by comparison of retention times with authentic standards on both polar and nonpolar capillary columns, microreaction-GC–EAD analyses, and field trapping tests. GC–EAD experiments showed that synthetic *E*11-16:Ald exhibited extraordinarily high electrophysiological activity, stimulating significant male antennal responses at as low as 10 fg. Traps baited with *E*11-16:Ald alone were attractive to males. Addition of 1 or 3% of its geometric isomer, *Z*11-16:Ald, to *E*11-16:Ald did not significantly increase trap captures, but an inhibitory effect was observed at the 10% level. The influence of two kinds of rubber septa on attraction was also evaluated. Male moth captures were higher in traps baited with red rubber septa than with gray rubber septa at 30–300-μg doses. Monitoring of adult flight activity with 3-μg doses of *E*11-16:Ald indicated at least three distinct flight periods throughout the 2003 season.

Key Words—Blueberry leafminer, *Caloptilia porphyretica*, sex pheromone, (*E*)-11-hexadecenal, gas chromatography–electroantennogram detection, microreactions, population monitoring, field trapping.

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INTRODUCTION

The blueberry leafminer, *Caloptilia porphyretica* Braun, has become a frequent pest of commercial highbush blueberries, *Vaccinium corymbosum* L., grown in Atlantic and Burlington Counties of New Jersey. This insect was first reported to infest azaleas in North Carolina (Braun, 1923). In recent years, severe infestations, with as high as 40–50% damaged leaves, have been observed in many locations in New Jersey.

Adult *C. porphyretica* are small moths with a wing span of ~1.5 cm. Eggs are deposited singly on the abaxial surface of the leaf, and at times more than one egg may be deposited on each leaf. Upon hatching, the larva bores through the lower wall of the egg and the leaf cuticle into the epidermis. The first two instars are sap feeders, whereas the later instars feed on leaf tissue within or outside the mine (Davis, 1987). The fourth instar larvae exit the mine and using silk, fold a single leaf into a symmetrical, triangular tent and feed on leaf tissue while remaining within the tent. Larvae leave the tents when mature and usually pupate on the abaxial leaf surface. This insect overwinters as a mature larva enclosed within a cocoon on senesced leaves on the soil surface. At present, there are no publications available on the biology and seasonal life history of *C. porphyretica*.

Although blueberry bushes can tolerate moderate leaf mining without loss of production, severe infestations may affect the plant vigor and cause yield loss. In addition to direct production loss, this insect can become a contaminant, especially in machine-harvested fruit, as the larvae drop from bushes along with the harvested fruit. The presence of larvae in harvested fruit can substantially increase sorting time on the packing lines and may result in a contaminated product.

The objectives of this study were to identify the sex pheromone of *C. porphyretica*, and to develop a pheromone lure for population monitoring. Sex-pheromone-baited traps will be useful in the study of the seasonal life history of *C. porphyretica*. The identification of the sex pheromone will enable possible future development of mating disruption and attract-and-kill technologies for managing *C. porphyretica* populations.

METHODS AND MATERIALS

Insects. Larvae of *C. porphyretica* were collected from commercial blueberry fields, near Hammonton, Atlantic County, in New Jersey during June–October of 2002. These larvae were individually reared in Chatsworth, NJ, in screen-top plastic containers (10 × 4 cm) in an incubator at 25°C and 16L:8D photoperiod. Moths that emerged were sexed and allowed to mate in a large screened wooden cage (1 × 1 × 1.5 m) with three to four 1-year-old blueberry plants, maintained in

a greenhouse at 20–27°C and 16L:8D photoperiod. Absorbent cotton moistened with 8% sugar water was provided as a food source for moths. Approximately 3 wk later, pupae were collected and kept individually in screen-top plastic containers in an incubator at 20°C and 16L:8D photoperiod until emergence. After emergence, moths were sexed and transferred to 15°C and 16L:8D photoperiod until used in experiments in Beltsville, MD.

Pheromone Gland Extractions. Pheromone gland extracts were obtained during photophase from seven groups of 5- to 20-d-old virgin females that were kept at 15°C and 16L:8D or previously used for effluvial collections (3, 15, 20, 30, 30, 100, and 150 females per group). A female abdomen was compressed gently until the ovipositor everted. The ovipositor was excised with microscissors into a conical glass vial containing ~100 μl methylene chloride/methanol (3:1). The glands were soaked for at least 2 hr at room temperature. Extracts were removed, and the glands were re-extracted with 100 μl methylene chloride/methanol. The combined solution was concentrated to ~20 μl under a nitrogen stream and kept at −30°C in a freezer.

Effluvial Collections. Volatiles were collected using four groups of 5- to 20-d-old virgin females (25, 30, 30, and 37 females per group) at room temperature. Moths were introduced separately into three 1-l, 4-necked glass containers (Zhang et al., 1994). Air was drawn into the container through 6–14 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA), and out of the container through two traps (15 cm × 1.5-cm o.d.) containing Super Q (200 mg each; Alltech Associates, Inc., Deerfield, IL) by vacuum (~1 l/min). Female moths were fed with 10% sugar solution on cotton balls and aerated continuously for 3–4 d at room temperature and 16L:8D photoperiod. The adsorbent traps were changed every 24 hr. Adsorbents were eluted with methylene chloride (4 × 0.5 ml); eluates (2 ml/each sample) were concentrated to ~20 μl under a nitrogen stream and stored at −30°C.

Microreductions. A 60-μl hexane solution containing 60 ng of *E*11-16:Ald or 10 female equivalent (FE) of gland extracts in a conical glass vial was treated with 20 μg of $NaBH_4$ (5 μl of 2-propanol solution, 4 mg/ml). The vial was swirled several times, and after 5 min, 5 μl of water was added and the mixture was agitated to hydrolyze the borate esters (Klun et al., 1982). The organic layer was transferred into another conical glass vial and analyzed by GC and GC–EAD.

Electrophysiological Recordings. The coupled gas chromatographic–electroantennographic detection (GC–EAD) system was as previously described (Zhang et al., 1997; Zhang and Polavarapu, 2003). A Hewlett-Packard 6890 gas chromatograph equipped with a 60 m × 0.25-mm i.d., 0.25-μm film-thickness DB-WAXETR capillary column (J&W Scientific Inc., Folsom, CA, 120°C for 2 min, then programmed to 250°C at 10°C/min and held for 10 min) or a 60 m × 0.25-mm i.d., 0.25-μm film-thickness DB-1 capillary column (J&W Scientific Inc., 100°C for 2 min, then programmed to 300°C at 10°C/min and held for

10 min) in the splitless mode with hydrogen as carrier gas (1.4 ml/min) was used for GC–EAD analysis.

Chemicals. (*E*)-11-Hexadecenal was purchased from the Pherobank, Wageningen, The Netherlands (>99% purity). All other synthetic pheromone standards had previously been synthesized and purified in our laboratory (Lynch et al., 1984), and stored in a −30°C freezer. Purities of chemicals were checked on a 60-m polar DB-WAXETR GC capillary column before preparing lures for the field study.

Field Tests. Red natural rubber septa (5 mm, Wheaton, NJ) and gray halobutyl rubber septa (5 mm, The West Company, NE) loaded with the desired rates of *E*11-16:Ald and a mixture of *EZ*-isomers in ~40 μl of hexane solution and 2 drops of butylated hydroxyltoluene (BHT) solution (10 mg/ml hexane) were used for field trials. The same amount of hexane was loaded on each kind of septum for the blank control. After loading, the solvent was allowed to evaporate in a fume hood for 30 min. Lures were wrapped in aluminum foil, stored in 20-ml plastic vials, and shipped by courier. Upon arrival in Chatsworth, NJ, the lures were kept in a freezer at −10°C until used.

All field tests were conducted in commercial blueberry fields near Hammonton, Atlantic County, and Chatsworth, Burlington County, NJ, using Pherocon 1C sticky traps (Trécé, Salinas, CA). Traps were arranged in a randomized complete block design with four or five replicates and 20–25-m intertrap distances within replicates. Replicates were separated by ~22 m (8 rows of blueberries). Traps were hung at crop canopy level ~150 cm above ground level, checked and moved by one position every 3–7 d in different tests, and the bottoms were replaced when catches exceeded 150 moths per trap. Caged live virgin females (2- to 4-d-old, 2 females per cage) were used as positive controls. Females were replaced with fresh insects after each trap check for the duration of the test.

Statistical Analysis. Data on trap catches from each test were square-root-transformed ($\sqrt{\chi} + 0.5$) or logarithm transformed ($\log\chi + 1$) to normalize the variance before analysis. Means were compared by either paired-samples *t*-test (2-tailed) or one-way analysis of variance (ANOVA) followed by Ryan–Einot–Gabriel–Welsch Range test (SPSS 10.0 for Windows) (George and Mallery, 2002) for significance at $\alpha = 0.05$.

RESULTS

Identification of the Antennal Stimulatory Component in Female Gland Extracts and Effluvial Collections. Coupled GC–EAD analyses of female gland extracts and effluvial collections demonstrated that antennae of male *C. porphyretica* consistently responded to a single compound (Figure 1A and B). The EAD-active peaks were observed at 10.97 min on a 60-m DB-WAXETR capillary column and at 12.78 min on a 60-m DB-1 capillary column. The amount of natural

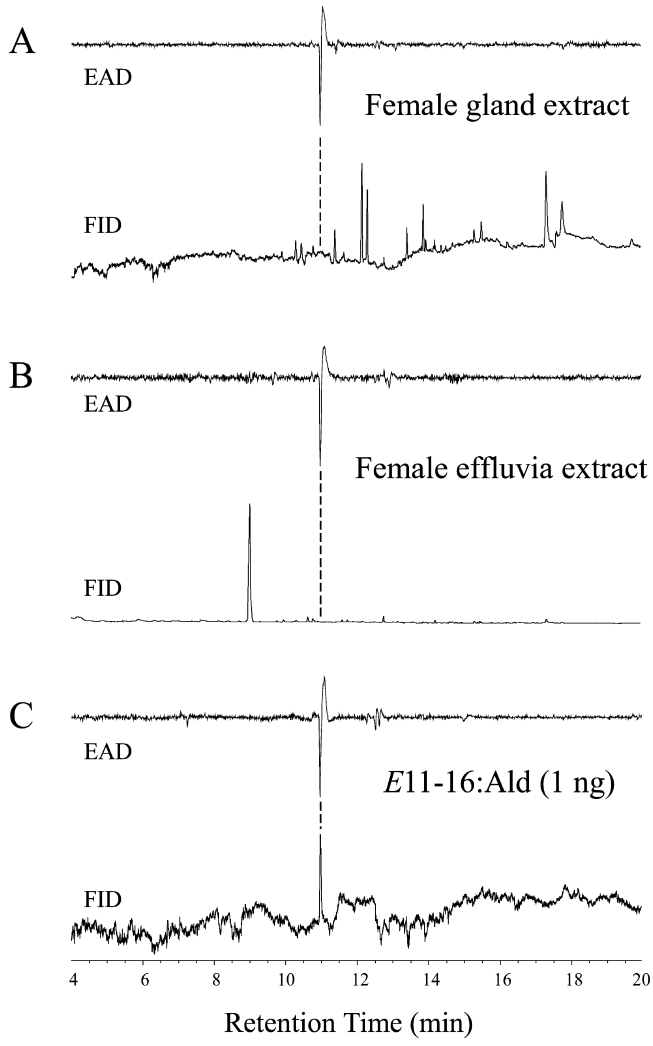

FIG. 1. Simultaneous FID and EAD responses of the antenna of an adult male *C. porphyretica* to (A) a pheromone gland extract (15 FE) from virgin female *C. porphyretica* (5- to 7-d-old); (B) effluvia (25 FE) trapped from virgin female *C. porphyretica* (5- to 20-d-old); and (C) synthetic *E*11-16:Ald (1 ng) on a DB-WAXETR column.

EAD-active component collected from female *C. porphyretica* was below the FID and MS detection thresholds, even with highly concentrated gland extracts (~150 FE). Therefore, identification of the EAD-active component relied on the comparison of the retention times of EAD-active compounds with authentic

standards on polar and nonpolar GC columns, and a microreaction followed by GC–EAD analysis.

In the genus *Caloptilia*, the only other species for which a female sex pheromone has been identified is the tea leafroller, *C. theivora* Walsingham (Ando et al., 1981, 1985). In this species, *E*11-16:Ald was identified as the main pheromone component, but 1–3% of *Z*11-16:Ald was found to be essential for optimum activity. Thus, we tested hexadecanal in GC-EAD analyses, and found that it stimulated strong EAD responses from male antennae. The retention times of 16:Ald at 10.67 min (polar column) and 12.93 min (non polar column) were slightly shorter than the EAD-active peak (10.97 min) on the DB-WAXETR column and slightly longer than the EAD-active peak (12.78 min) on the DB-1 column, suggesting that the pheromone component could be an unsaturated C_{16} aldehyde. The low GC–EAD responses and nonmatching retention times of C_{14} and C_{16} unsaturated acetates ruled out the possibility of acetates being the pheromone candidates (Table 1). When a variety of monounsaturated aldehydes were subjected to GC analyses, EAD-active peaks matched only with the GC retention times of *E*11-16:Ald on both capillary columns (Table 1), and the strong antennal activity of *E*11-16:Ald was confirmed with an authentic standard (Figure 1C).

Antennae of female *C. porphyretica* responded to all the compounds tested (Table 1). When 30 ng of synthetic *E*11-16:OH (>99.9% pure) was subjected to GC–EAD analysis, strong EAD responses were elicited not only from the

TABLE 1. RETENTION TIMES OF EAD-ACTIVE COMPOUND OBTAINED FROM EFFLUVIA AND GLAND EXTRACTS OF FEMALE *C. porphyretica* AND SYNTHETIC STANDARDS

Compounds	Rentention time (min)		EAD activity in 10 ng of loading (mV)
	DB-WAXETR	DB-1	
From females			
(*E*)-11-16:Ald	10.97	12.78	
Synthetic			
16:Ald	10.67	12.93	1.30 (±10.0% SD, $n = 3$)
(*Z*)-7-16:Ald[a]	10.93	12.70	
(*Z*)-9-16:Ald[a]	10.95	12.74	
(*E*)-11-16:Ald	10.97	12.78	2.30 (±10.1% SD, $n = 3$)
(*Z*)-11-16:Ald	11.04	12.81	0.27 (±5.8% SD, $n = 3$)
(*E*)-11-16:OH	13.08	13.48	0.67 (±11.6% SD, $n = 3$)
(*E*)-11-14:Ac	10.62	12.81	0.30 (±10.0% SD, $n = 3$)
(*Z*)-11-14:Ac	10.74	12.87	0.17 (±0.58% SD, $n = 3$)
(*Z*)-12-14:Ac	11.07	13.06	0.30 (±10.0% SD, $n = 3$)
(*E*)-11-16:Ac	12.46	14.71	0.23 (±5.8% SD, $n = 3$)

[a] Compounds not tested by EAD.

*E*11-16:OH (Figure 2A, peak 2), but also from a FID-undetectable residual *E*11-16:Ald contaminant (Figure 2A, peak 1), indicating that male *C. porphyretica* antennae were extremely sensitive to the *E*11-16:Ald.

The identity of *E*11-16:Ald was further verified by treatment of the female gland extracts with $NaBH_4$ to convert the aldehyde to the corresponding alcohol.

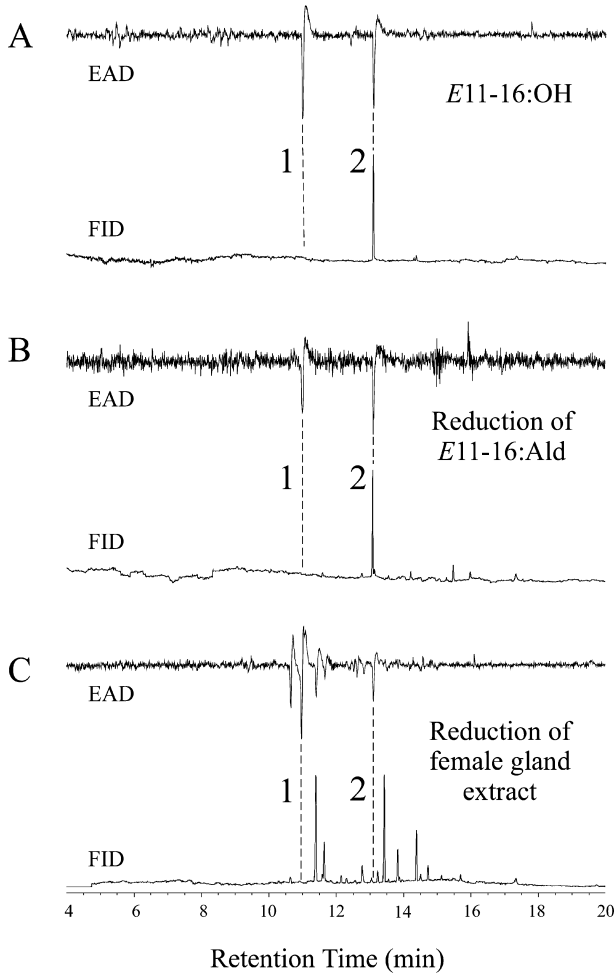

FIG. 2. Simultaneous FID and EAD responses of an adult male *C. porphyretica* antenna to (A) 30 ng of synthetic *E*11-16:OH; (B) $NaBH_4$-reduced *E*11-16:Ald (~10 ng); and (C) $NaBH_4$-reduced gland extracts (5 FE) on a DB-WAXETR column. Identifications of peaks: **1**, *E*11-16:Ald; **2**, *E*11-16:OH.

GC–EAD of the reduced gland extract showed the presence of a new EAD response at the retention time of *E*11-16:OH on both capillary columns (Figure 2C), providing supporting evidence that the corresponding aldehyde, *E*11-16:Ald, was present in the original extract. EAD peak 1 was elicited by traces of unreacted *E*11-16:Ald. Treatment of 30 ng of synthetic *E*11-16:Ald with $NaBH_4$ resulted in similar GC–EAD responses (Figure 2B). Because even trace amounts of residual *E*11-16:Ald in reaction mixtures still evoked large EAD responses, other microreactions were not attempted. In total, the various pieces of information provide strong evidence that *E*11-16:Ald is a female sex pheromone component of *C. porphyretica*.

GC–EAD experiments were conducted with antennae of male *C. porphyretica* challenged with various standards. Among monounsaturated C_{14} and C_{16} standards, aldehydes elicited much stronger EAD responses than other candidate pheromone components (Table 1). In particular, male *C. porphyretica* antennae exhibited ability to discriminate the *Z*- and *E*11-16:Ald isomers. Antennae responded strongly to traces of *E*11-16:Ald when >99% pure *Z*11-16:Ald was subjected to GC–EAD analysis (Figure 3). The GC–EAD experiments also showed that synthetic *E*11-16:Ald elicited unusually strong electrophysiological activity, resulting in significant male antennal responses at doses as low as 10 fg (Figure 4).

Field Trapping Tests. Traps baited with 3 μg of *E*11-16:Ald were remarkably attractive to male *C. porphyretica* in the field. The captures in *E*11-16:Ald-baited traps were influenced by climatic factors during the flight season and other factors, such as insecticide applications. However, trap captures indicated that there were

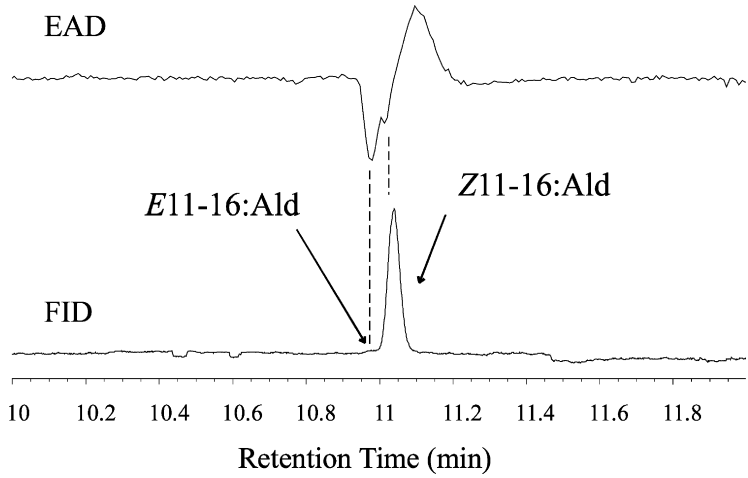

FIG. 3. Simultaneous FID and EAD responses of an adult male *C. porphyretica* antenna to 10 ng of synthetic *Z*11-16:Ald on a DB-WAXETR column.

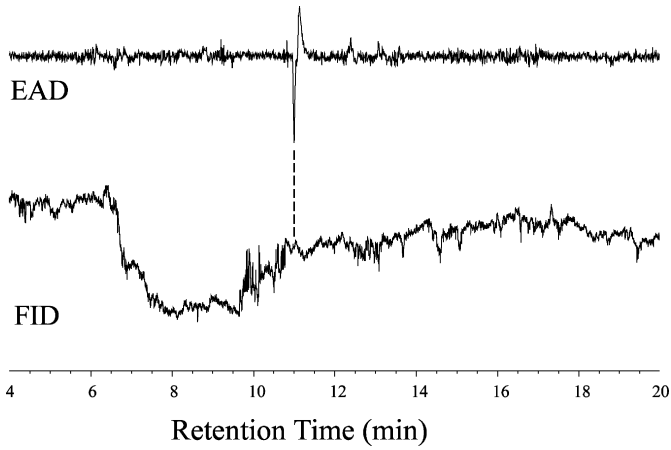

FIG. 4. Simultaneous FID and EAD responses of an adult male *C. porphyretica* antenna to 10 fg of synthetic *E*11-16:Ald on a DB-WAXETR column.

at least three distinct peaks (May 12, July 25, and September 10) separated by periods (~1–2 wk) with few male catches. Considering the time required for egg, larval, and pupal development, there appears to be at least three distinct generations each season (Figure 5).

Initial field tests conducted from July 1 to August 19, 2003, demonstrated that moth catches (total number of male *C. porphyretica* captured was 12,264) were similar in traps baited with gray or red natural rubber septa loaded with 3 μg of *E*11-16:Ald ($t = -1.104$, df = 3, $P = 0.350$). *E*11-16:Ald dose dramatically affected the trap catch of *C. porphyretica*. *E*11-16:Ald doses of 30–100 μg on gray rubber septa resulted in higher trap captures than 0.5–10-μg doses (Figure 6).

To investigate further the efficacy of gray and red rubber septa, we compared these septa loaded with 30–1000 μg of pheromone along with traps baited with two virgin females. Red rubber septa loaded with 1000-μg doses attracted more males than 100-μg and lower doses, whereas catches with 300-μg doses were intermediate (Figure 7). However, with gray rubber septa, moth catches were significantly higher with 1000-μg doses than any other doses tested. Traps baited with gray or red rubber septa loaded with 1000 μg of *E*11-16:Ald attracted similar numbers of males as traps baited with two virgin females (Figure 7). Data from this experiment also demonstrated that, except at the highest dose (1000 μg/septum), red rubber septum lures attracted more males than gray rubber septa loaded with the same doses.

In the final field test, we evaluated the effect of addition of 1–10% of *Z*11-16:Ald to *E*11-16:Ald (Figure 8). Addition of 1 and 3% of the *Z*-isomer to

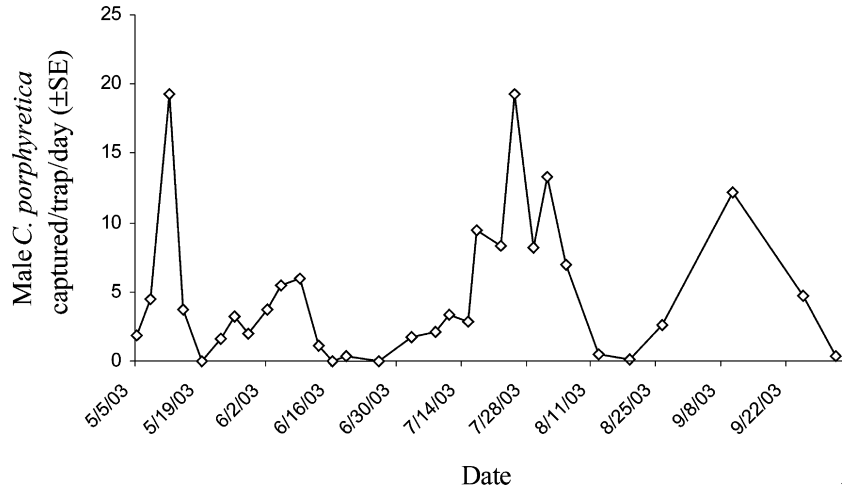

FIG. 5. The flight activity of *C. porphyretica* monitored by Pherocon 1C traps baited with 3 μg *E*11-16:Ald (5/1–7/3 using ~95% pure compound on gray rubber septa and 7/3–10/2 using ~98% pure compound on red rubber septa). The trial was conducted from May 1 to October 2, 2003. Total number of male *C. porphyretica* captured was 2481.

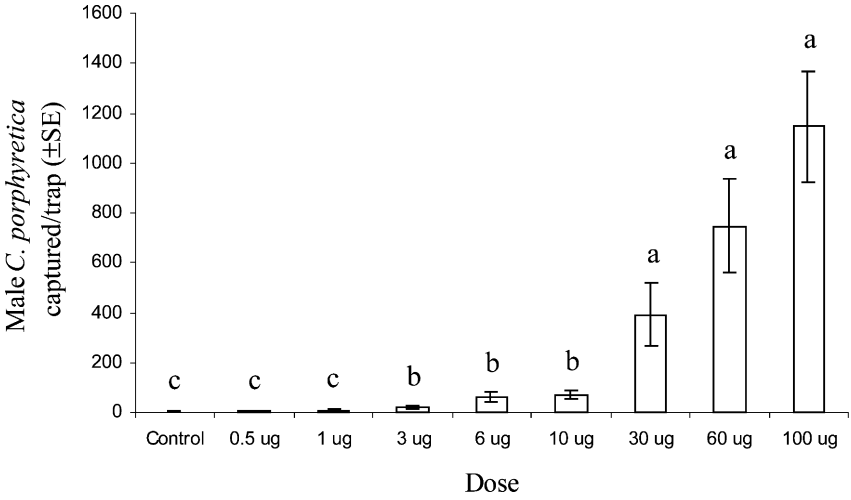

FIG. 6. Results of *C. porphyretica* pheromone dose–response field tests on gray rubber septa. The trial was conducted from July 1 to August 1, 2003. Total number of male *C. porphyretica* captured was 12,264. Bars superscripted by different letters are statistically different (logarithm transformed, $N = 5$, $F_{8,36} = 49.67$, $P < 0.05$).

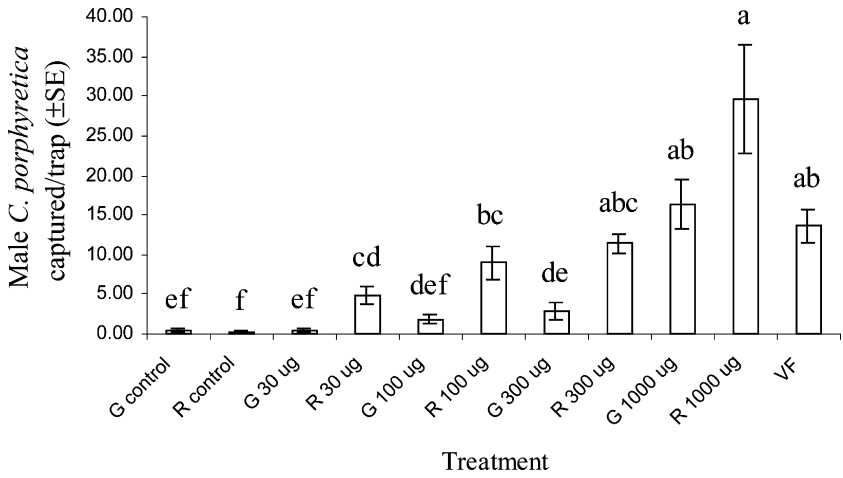

FIG. 7. *C. porphyretica* males captured in traps baited with two virgin females and different amounts of *E*11-16:Ald on two kinds of rubber septa. The trial was conducted from August 29 to September 23, 2003. Total number of male *C. porphyretica* captured was 452. Bars superscripted by different letters are statistically different (logarithm transformed, $N = 5$, $F_{10,44} = 28.07$, $P < 0.05$). G = gray halo-butyl rubber septa, R = red natural rubber septa, and VF = two virgin females.

*E*11-16:Ald (100-μg dose) did not increase trap catches compared with traps baited with 100 μg of *E*11-16:Ald alone. However, as the proportion of *Z*-isomer increased to 10%, male captures decreased compared with captures in traps baited with 0, 1, and 3% of the *Z*-isomer.

DISCUSSION

On the basis of GC-EAD analyses of gland extracts, effluvial collections, microreaction of gland extracts, and field trapping studies, we conclude that *E*11-16:Ald is the EAD-active compound produced by female *C. porphyretica*. Although *Z*11-16:Ald was also electrophysiologically active, addition of 1–3% of this compound to *E*11-16:Ald had no effect on trap captures, suggesting that this compound may not be part of the sex communication system of *C. porphyretica*.

Virgin females under laboratory conditions called during the first 2 hr of the scotophase. The EAD responses of gland extracts collected during photophase were identical to effluvial collections made over a 3-d period, suggesting that glands during photophase contained the same active compound. One possible reason that the amounts of pheromone recovered from gland extracts were so low is that the moths were dissected outside their normal calling period.

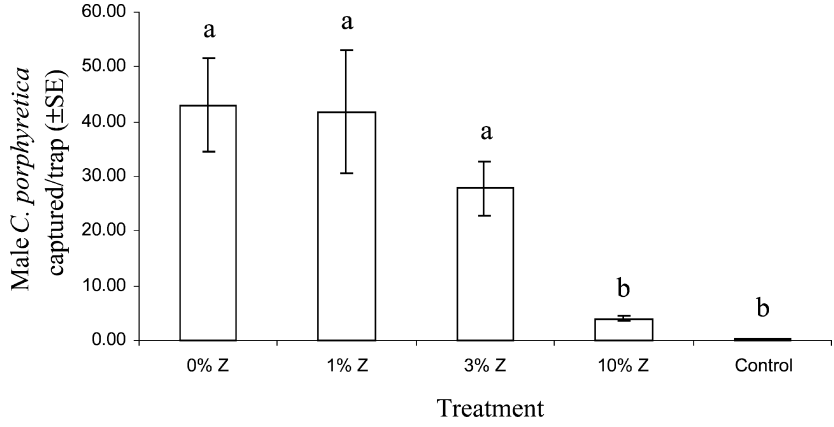

FIG. 8. Effect of addition of Z11-16:Ald to E11-16:Ald on captures of *C. porphyretica* male moths. Amounts of pheromone were 100 μg/septum. Percentage of Z11-16:Ald on the x-axis represents additional amounts of Z11-16:Ald added to 100 μg of E11-16:Ald. The trial was conducted from August 29 to September 23, 2003. Total number of male *C. porphyretica* captured was 584. Bars superscripted by different letters are statistically different (square-root-transformed, $N = 5$, $F_{4,20} = 21.71$, $P < 0.05$).

E11-16:Ald was first identified as a sex attractant for male moths in several lepidopteran families by systematic field screening tests (Ando et al., 1981). This compound has also been reported as a pheromone component of crambid moths, *Diaphania nitidalis* (Klun et al., 1986), and *D. hyalinata* (L.) (Raina et al., 1986), the sphingid moth *Deilephila elpenor* (Bestmann et al., 1992), and the pyralid moths, *Cryptoblabes gnidiella* (Anshelevich et al., 1993) and *Neoleucinodes elegantalis* (Cabrera et al., 2001).

In Lepidoptera, the use of a species-specific blend of pheromone components for sexual communication is more pervasive than the use of single compounds (Arn, 2000). However, in the family Gracillariidae, single-component pheromones appear to be more common. For example, (8E,10E)-tetradecadienal has been reported as a sex pheromone component in *Acrocercops* spp. (Ando et al., 1987). Its geometric isomer, (8E,10Z)-tetradecadienal, is a sex pheromone component of horse chestnut leafminer, *Cameraria ohridella* Deschka & Dimic (Svatos et al., 1999; Francke et al., 2002; Kalinová et al., 2003). *Gracillaria elongella* Linnaeus releases (10Z, 12E)-hexadecadienal (Arn, 2000) and *G. syringella* Fabricius emits (E)-11-tetradecenal that attracts males (Booij and Voerman, 1985). Furthermore, (4E,10E)-dodecadienyl acetate has been discovered in female pheromone gland extracts of tentiform leafminer, *Phyllonorycter mespilella* (Huebner) (Gries et al., 1993). Female *P. platani* emit (Z)-10-tetradecenyl acetate for mate location (Subchev et al., 2003), and the same compound has been found from abdominal

tip extracts of the tentiform leafminer moth *P. ulmifoliella* (Mozuraitis et al., 1997). Finally, (*E*)-10-dodecenyl acetate has been used for monitoring as well as mating disruption of spotted tentiform leafminer, *P. blancardella* (Fabr.) (Trimble and Tyndall, 2000).

Gray halobutyl rubber septa were reported to be better for stabilizing unsaturated pheromone components than red natural rubber septa (Brown and McDonough, 1986). However, on the basis of two different field experiments, we did not see significant differences in moth catches in traps baited with gray and red rubber septa loaded at very low (3-μg) or very high (1000-μg) rates (Figure 7), but red rubber septa attracted significantly more moths at 30–300-μg doses compared with gray rubber septa at the same doses (Figure 7).

Within limits trap catches usually increase with increasing pheromone loading (Carde and Elkinton, 1984; Polavarapu and Seabrook, 1992; Facundo et al., 1994). When doses were increased from 0.5 to 100 μg on gray septa, mean trap capture increased over three orders of magnitude and no inhibitory effects were observed (Figure 6). The larger trap catches observed with increasing doses of *E*11-16:Ald in different experiments (Figures 6 and 7) might be attributed to expanded active space of the trap and overlap of active spaces, causing male moths to preferentially seek traps baited with higher doses upwind (Wall and Perry, 1987). Furthermore, moth captures were comparable in traps baited with two virgin females and traps baited with red rubber septa baited with 100–1000-μg doses, indicating the suitability of doses in this range for population monitoring.

Overall, the electrophysiological, chemical, and behavioral data reported here all support the identity of *E*-11-16:Ald as the main pheromone component of *C. porphyretica*. (*E*)-11-Hexadecenal at doses of 100–300 μg on red natural rubber septa should provide an effective lure for monitoring populations of this insect.

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